Regulation of the dnaK Operon of Streptomyces coelicolor A3(2) Is Governed by HspR, an Autoregulatory Repressor Protein

GISELDA BUCCA, ZOË HINDLE,† AND COLIN P. SMITH*

Department of Biochemistry and Applied Molecular Biology, U.M.I.S.T., Manchester, M60 1QD, United Kingdom

Received 3 February 1997/Accepted 29 July 1997

Free-living organisms generally respond to an injury caused by exposure to a physicochemical or metabolically stressful agent by inducing the synthesis of a set of proteins, collectively called heat shock proteins (Hsps), which help to prevent or repair damage caused by partially denatured proteins that may or may not have formed insoluble aggregates (15, 19). Some Hsps are molecular chaperones which prevent the aggregation of partially unfolded proteins and help them to fold into their native structures (reviewed in reference 18), while others are proteases, such as Lon and Clp, which degrade unfolded proteins that have formed insoluble aggregates (see, e.g., reference 16). Some Hsps, such as the molecular chaperones Hsp70 (DnaK) and Hsp60 (GroEL), also fulfill crucial roles under normal physiological conditions by assisting in the folding of newly synthesized polypeptides (see, e.g., reference 18). Although these chaperones rank as perhaps the most highly conserved proteins in nature, diverse regulatory mechanisms have evolved in different organisms for controlling their synthesis. In Escherichia coli the coordinated induction of the heat shock regulon is achieved by increasing the cellular concentration of two alternative σ factors: σ32 activates heat shock genes following a mild heat shock (9, 14, 45), while σ32 activates transcription under conditions of severe heat shock (31, 33). This positive mode of regulation is moderately conserved in gram-negative bacteria, particularly in the subgroup of purple bacteria to which E. coli belongs (27). It has recently become clear, however, that many bacteria utilize a repressor/operator system for controlling heat shock genes. The most widespread negative-control system involves the binding of a specific repressor, HrcA (formerly called OrfA, or Orf39), to a highly conserved operator element designated CIRCE (see, e.g., references 3, 4, 32, 35, 37, 44, and 46). Some gram-negative species have recruited both the HrcA/CIRCE and alternative σ factor control systems for controlling different heat shock genes (3), while others have combined both systems for modulating the expression of specific operons (1, 32). With the current exception of the dnaK operons of actinomycetes, the dnaK and groE operons of all gram-positive bacteria studied appear to be regulated by the HrcA/CIRCE system (reviewed in reference 38).

We are studying the regulation of the heat shock response in the model streptomycte Streptomyces coelicolor A3(2). Streptomyces are members of the ancient order Actinomycetales, which also includes the mycobacteria, and they are part of the phyllogenetically distinct high-G+C gram-positive group. We have characterized the dnaK operon of S. coelicolor and studied its regulation (6, 7). The S. coelicolor dnaK operon promoter region does not contain a CIRCE element (6), whereas its two groE operons each contain two promoter-proximal CIRCE sequences (12, 13). The dnaK operon encodes four proteins, DnaK, GrpE, DnaJ, and HspR (see Fig. 1). HspR represents a novel heat shock protein which binds to three inverted repeat sequences (IR1 to IR3) in the dnaK operon promoter region (6). There is no sequence similarity between the HspR/IR and HrcA/CIRCE systems, and HspR appears, so far, to be unique to the actinomycete bacteria. We previously speculated that HspR functions as a repressor of the S. coelicolor dnaK operon (6). This role is confirmed in the present study. We show that disruption of hsrP leads to high-level constitutive expression of the dnaK operon but does not significantly perturb heat shock regulation of the two groE operons. HspR titration experiments were also conducted and provided confirmation that HspR functions as a negative autorepressor. We also provide additional biochemical evidence that HspR directly regulates transcription of the dnaK operon.
MATERIALS AND METHODS

Bacterial strains and plasmids. S. coelicolor A3(2) strain MT1110 (13a) is a SCP1-SCP2 derivative of the prototrophic wild-type strain MT111 (23). Streptomyces lividans 1326 is the prototrophic wild-type strain (23). E. coli XL1-Blue (Strain was used as the general cloning host, and E. coli BL21 (DE3) pLysS) (F’ompT hsdSB (rK mK) gall dcm (ADE3) PlysS (Cm)) (43) was used for overproduction of HspR. E. coli ET12567 (F’ dam-13:Trsp dcm-hsdM HsdS recF143 zjj-202:TraT7 rplM15) (25) was used for propagating plasmid DNA free of dam activity and hsd methylation.

E. coli plasmid pG4.2, which contains the complete dnak gene and 0.3 kb of the gpe gene of S. coelicolor (7), was used as the source of dnak probes for Southern analysis and S1 nuclease mapping experiments. E. coli plasmid pG4.2, containing the complete 4.5-kb hspR operon promoter fragment (6), was isolated to identify the HspR-binding sites on a 200-bp SacII-NcoI fragment. The latter, encompassing nucleotides −65 to +35 relative to the transcription start site, was used as the template for the HspR titration studies; the fragment was first blunt ended and subcloned into the SacI site of pMT3010 (29), a multicopy pHJ101 derivative (29), was used for subcloning the dnak promoter for HspR titration studies. Plasmids pAM102 and pAM120 (12) were used as the sources of DNA probes for transcriptional analysis of groEL2 and groEL1 expression, respectively.

Construction of the hspR-disrupting plasmid pMT3302. Plasmid pHJ309, a derivative of an E. coli plasmid containing the thiostrepton resistance gene (tss) (36), was used as the delivery vector for constructing the hspR null mutant. The hspR gene was disrupted by homologous recombination in the E. coli plasmid pHJ309 as described in reference 4, and the resulting DNA fragment was used to replace the intact hspR gene in the E. coli strain. The resulting E. coli strain was grown on the appropriate selective medium to verify the disruption of the hspR gene. The resulting E. coli strain was then used to transform S. coelicolor A3(2) strain MT1110 (13a) by the tri-parental matings method described in reference 10. The resulting S. coelicolor (MT1110 + pMT3302) strain was then grown on the appropriate selective medium to verify the integration of the hspR-disrupting plasmid, and the resulting S. coelicolor strain was used for further experiments.

RESULTS AND DISCUSSION

Construction of an hspR null mutant of S. coelicolor. In order to test the role of HspR in regulation of the dnak operon, the hspR gene was disrupted by cloning the hygromycin resistance gene into the unique SacII site at the start of the hspR coding sequence (Fig. 1). The resulting S. coelicolor was transformed with the resulting plasmid, pMT3302, which does not replicate in Streptomyces (see, e.g., reference 20). Generally 1 to 2 transformants/µg were obtained with covalently closed circular DNA, whereas approximately 100/µg were obtained with NaOH-denatured plasmid DNA. All transformants obtained were doubly resistant to hygromycin and thiostrepton, indicating that in all cases the plasmid had integrated by a single homologous recombination event via one of the two chromosomal sequences flanking the hyg gene. With pMT3302, an integration event via the 1-kb segment upstream from hyg would disrupt the dnak operon at codon 8 of hspR, while integration via the downstream 0.8-kb segment would re-form an intact operon (since the latter fragment is not internal to the transcription unit [see, e.g., reference 11]). Of 10 transformants analyzed by Southern blotting, 2 had integrated via the upstream segment to generate hspR null mutants (designated MT1151 and MT1153) while 8 had integrated nonhomogenously via the downstream segment; two of the latter hspR integrants, MT1152 and MT1154, were used as controls in this study. The single-crossover integrants MT1151 through MT1154 were found to be highly stable and were therefore used for the transcriptional studies.
Analysis of heat shock gene expression in hspR null mutants. RNA was isolated from surface-grown cultures of MT1151 through MT1154 cultivated at 30°C and from duplicate cultures heat shocked at 42°C for 15 min. In order to directly compare the effect of the hspR mutation on transcription of the dnaK, groESL1, and groEL2 operons, the same four pairs of RNA preparations were subjected to S1 nuclease analysis with each of the operon-specific probes. The results demonstrate clearly that dnaK transcription is derepressed in an hspR null mutant, with the level of transcription at 30°C being comparable to the induced level in heat-shocked cultures (Fig. 2). This indicates that HspR is principally responsible for negatively regulating transcription of the dnaK operon. In contrast, transcriptional regulation of groESL1 was unaffected in an hspR null mutant. Interestingly, the basal level of transcription of groEL2 at 30°C was slightly elevated (approximately two- to threefold) in the hspR null mutant. It is considered that this partial increase in groEL2 transcription level at ambient temperature is an indirect consequence of disrupting hspR, resulting either from the enhanced synthesis of the DnaK chaperone machine or other, as yet unidentified, components of the HspR regulon. It may be significant in this context that groEL2, but not groEL1, appears to be essential for viability in another streptomycete, Streptomyces albus (39). In a parallel biochemical study, we found that purified His-HspR protein failed to retard templates containing either the groESL1 or the groEL2 promoter region in gel shift experiments, whereas it efficiently retarded a dnaKp template (data not shown). This result is consistent with the above transcriptional analyses, and we conclude that neither of the groE operons forms part of the HspR regulon.

Attempted titration of HspR by cloning HspR-binding sites at high copy number. HspR represses transcription from the dnaK operon promoter. Therefore, if the level of synthesis of HspR is determined principally by the level of transcription from the dnaK operon promoter, then it would follow that expression of the dnaK operon is controlled by an HspR-mediated negative autoregulatory feedback loop. If this is the case, then it would not be possible to deregulate the operon by titrating away HspR by, for example, introducing multiple copies of its binding site into the cell; titration of the repressor would stimulate the production of more repressor and would therefore maintain repression of the operon. This prediction was tested by cloning the three HspR-binding sites from the dnaK promoter region (IR1 through IR3 [6]) into a multi-copy plasmid to form pMT3301 (see Materials and Methods) and assessing the effect of pMT3301 on regulation of dnaK
transcription. In this experiment, two independent *S. lividans* clones were analyzed, and plasmid copy number was determined from the same cultures used for RNA isolation; copy number was assessed by comparing the relative hybridization signals from the plasmid-borne *dnaK* promoter and region and the corresponding chromosomal sequence. Despite their containing approximately 50 additional HspR-binding sites per chromosome, regulation of the chromosomal *dnaK* operon in these clones was unaffected and heat shock regulation of the plasmid-borne *dnaK* promoters was maintained (Fig. 3). (*S. lividans* and *S. coelicolor* are extremely closely related strains, and for the purposes of this study, they are considered the same; their respective *dnaK* operons have an identical restriction map, and heat shock regulation appears identical in both strains.) Comparable results were obtained by Yuan and Wong (44) in a titration experiment with *B. subtilis* which used a CIRCE-containing multicopy plasmid, showing that the presence of the His tag did not alter the DNA-binding activity of the protein (data not shown). It was not possible, however, to purify the His-HspR directly from the cell extracts by metal affinity chromatography; the protein was not retained on a nickel (Ni-nitrilotriacetic acid) column, and it was considered likely that the amino-terminal His tag was sequestered within the tertiary structure of the protein. Therefore, in order to purify the His-HspR, chromatography was repeated under denaturing conditions (in the presence of 8 M urea), and His-HspR fractions of 90 to 95% purity were subsequently refolded as described in Materials and Methods. The activity of the renatured His-HspR was assessed by competition gel retardation studies (see, e.g., reference 6) and was shown to bind specifically to the *dnaK* promoter fragment (data not shown).

**Repression of dnaK transcription in vitro by His-HspR.** In vitro transcription experiments were conducted in the presence and absence of His-HspR to assess whether the protein, in isolation, could repress transcription from the *dnaK* promoter. In parallel with the *dnaKp* reactions, a negative-control template was used, containing the glycerol operon (*gyl*) promoter region of *S. coelicolor* (41). HspR was shown to virtually abolish transcription from the *dnaK* promoter (Fig. 4). Although HspR also reduced transcription from the control *gyl* template, the repression ratio was substantially lower. The latter repression is considered to result from interference caused by a degree of nonspecific binding of refolded or partially folded His-HspR. In a separate study, the *dnaK* promoter was used as a control template in analyzing GylR-mediated repression of *gyl* transcription in vitro (21); in that study, purified GylR protein was shown to cause complete repression of transcription from the *gyl* template, but it also repressed, to a much lesser extent, transcription from the *dnaK* template. Although the results from the in vitro transcription experiments cannot alone be used as unequivocal evidence that HspR represses the *dnaK* operon, they are entirely consistent with the results of the hspR disruption experiments and with our previous observation that HspR binds specifically to three sites within the *dnaK* promoter.

**Concluding remarks.** Our studies indicate that the *S. coelicolor* HspR protein represses transcription of the *dnaK* operon...
and regulates its own synthesis. Moreover, the transcriptional studies of hspR null mutants indicate that HspR does not regulate the two groE operons of *S. coelicolor*, although loss of HspR did appear to slightly enhance the basal level of transcription of groEL.

In low-G+C gram-positive bacteria, such as *B. subtilis*, the *dnaK* and *groE* operons are members of the same regulon, under the control of the HrcA/CIRCE system (see, e.g., references 35 and 44). Although streptomycete *dnaK* operons are not subject to such control, there is strong circumstantial evidence that their *groE* operons are regulated by the HrcA/CIRCE system (see, e.g., reference 13). Thus, streptomycetes have recruited two separate repressor/operator systems for controlling the synthesis of the two key chaperone machines. This observation suggests that there is not a strict requirement for a particular stoichiometry of the two systems within the cell, and it is consistent with the current notion that they form part of a complex network for assisting protein folding rather than an obligatory pathway, where the two systems act successively (see, e.g., reference 8). HrcA and HspR share the common feature of functioning as negative autoregulators (reference 44, see, e.g., reference 8). HrcA and HspR share the common feature of functioning as negative autoregulators (reference 44).

ACKNOWLEDGMENTS

We thank Rob Beynon, Keith Chater, and Fiona Flett for helpful discussions, and we thank Philippe Mazodier for providing plasmids pAMD12 and pAMD20.

This work was partly supported by a grant from the Biotechnology and Biological Sciences Research Council (United Kingdom), and at the beginning of this work, G.B. was partly supported by a fellowship from the University of Palermo (Palermo, Italy).

REFERENCES

13a.Frazer, C. Personal communication.
24. Lydiate, D. J. Personal communication.


